Kinetic benefits and thermal stability of orotate phosphoribosyltransferase and orotidine 5'-monophosphate decarboxylase enzyme complex in human malaria parasite Plasmodium falciparum

Panan Kanchanaphum, Jerapan Krungkrai *

Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

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A B S T R A C T

We have previously shown that orotate phosphoribosyltransferase (OPRT) and orotidine 5'-monophosphate decarboxylase (OMPDC) in human malaria parasite Plasmodium falciparum form an enzyme complex, containing two subunits each of OPRT and OMPDC. To enable further characterization, we expressed and purified P. falciparum OPRT–OMPDC enzyme complex in Escherichia coli. The OPRT and OMPDC activities of the enzyme complex co-eluted in the chromatographic columns used during purification. Kinetic parameters (Km, kcat and kcat/Km) of the enzyme complex were 5- to 125-folds higher compared to the monofunctional enzyme. Interestingly, pyrophosphate was a potent inhibitor to the enzyme complex, but had a slightly inhibitory effect for the monofunctional enzyme. The enzyme complex resisted thermal inactivation at higher temperature than the monofunctional OPRT and OMPDC. The result suggests that the OPRT–OMPDC enzyme complex might have kinetic benefits and thermal stability significantly different from the monofunctional enzyme.

Introduction

Plasmodium falciparum is a major causative agent for malaria, responsible for over a hundred million clinical cases per year. The malaria parasite is totally dependent on de novo synthesis of pyrimidine nucleotide [1], whereas the mammalian host cells obtain the pyrimidine nucleotides from both de novo and salvage pathways [2]. Six enzymatic reactions are involved in the de novo pathway. In the final two reactions, uridine 5'-monophosphate (UMP) synthesis requires the addition of ribose 5-phosphate moiety from 5-phosphoribosyl-1-pyrophosphate (PRPP) to orotate by orotate phosphoribosyltransferase (OPRT) to form orotidine 5'-monophosphate (OMP) and pyrophosphate (PPi), where OMP is thereafter decarboxylated to UMP by OMP decarboxylase (OMPDC).

For most prokaryotes and yeast [3,4], the OPRT and OMPDC enzymes are encoded by two separate genes, while in majority of multicellular eukaryotes, the genes for both enzymes are fused into a single gene, named UMP synthase, which bears two different catalytic domains [2,5,6]. The bifunctional UMP synthase is also found in kinetoplastid parasites, e.g., Trypanosoma cruzi and Leishmania mexicana [7,8]. The biochemical characteristics of the de novo pathway in P. falciparum have been studied [1], and the inhibitors targeting the enzymes of the pathway that are lethal to the cultured parasite have been identified [9–11].

The OPRT and OMPDC genes of P. falciparum are located on different chromosome [12,13], encoding proteins with 289 amino acids for PfOPRT and 323 amino acids for PfOMPDC [14,15]. Both enzymes exist as an enzyme complex having a molecular mass of 140 kDa, with two molecules each of PfOPRT and PfOMPDC [14,16]. The PfOPRT–PfOMPDC enzyme complex is unique to malaria parasites, and thus inhibition of the UMP synthesis may provide new antimalarial drugs [16].

Here, we report the expression, purification, kinetic, inhibition and thermal stability of the PfOPRT–PfOMPDC enzyme complex from Escherichia coli. We show that the enzyme complex has significant differences in kinetic properties, inhibitory constants and thermal stability from PfOPRT and PfOMPDC monofunctional enzyme.

Materials and methods

Materials. Restriction enzymes, chemical reagents, chromatographic apparatus and other molecular biology supplies were purchased from Amicon, Bio-Rad, GE Healthcare, Promega, Quiagen, Roche and Sigma.

Co-transformation of PfOPRT and PfOMPDC. Competent E. coli TOP10 cells were transformed with PfOPRT-pQE30Xa and...
PFOMPDC-pTrcHisA plasmids constructed as described [14,15]. The cells were grown in LB medium at 37 °C, and induced for 18 h at 18 °C with 1 mM isopropyl β-D-thiogalactopyranoside, harvested by centrifugation at 8000g then washed twice with cold 1× phosphate buffer saline. Cells were stored at −70 °C until use.

Expression and purification of recombinant proteins. The Ni²⁺-NTA agarose affinity purification was done essentially as described [15]. The eluent from the Ni²⁺-NTA column was added onto Hi Trap Q HP anion-exchange column equilibrated with 50 mM Tris–HCl pH 8.0 and eluted using 50 mM Tris–HCl pH 8.0 and 250 mM NaCl. The eluted fractions from the Hi Trap Q HP column containing both PfOPRT–PfOMPDC were then concentrated by Centricon™10 device. Preparations of purified recombinant monofunctional enzymes were as previously described using E. coli TOP10 as host cells [14,15].

Enzyme assays. Enzymatic activities of OPRT and OMPDC were monitored by spectrophotometric method as described [16].

Enzyme kinetics and inhibition studies. Kinetic analyses of the PfOPRT–PfOMPDC enzyme complex were determined by using the Hi Trap Q HP column purified enzyme complex (>95% pure as observed by SDS–PAGE). $K_m$ and $V_{max}$ values were determined by triplicate measurement of initial velocities with at least five substrate concentrations. The monofunctional enzyme was also included for all enzyme activities. Enzymes concentrations used for all studies were 14–16 nM.

For OPRT assay, the reaction mixture contained 25–250 μM orotate, 25–250 μM PRPP, 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM MgCl₂, and 250 μM DTT. For OMPDC, the reaction mixture contained 20–125 μM OMP, 50 mM Tris–HCl pH 8.0, 150 mM NaCl, and 250 μM DTT. The catalytic efficiency ($k_{cat}/K_m$) values of both OPRT and OMPDC in the enzyme complex were also calculated. Inhibition studies were performed separately for both monofunctional enzyme and PfOPRT–PfOMPDC complex. Kinetic data of initial velocities and inhibitions were fitted to the equations for competitive, non-competitive, and un-competitive inhibitors [17] using the method of Cleland [18].

Thermal stability test. Thermal stability of the enzymes was assayed at various temperatures (37–60 °C) for 30 min. Enzyme activities were determined after quick cooling for 10 min. The temperature at half enzymatic activity ($T_{1/2}$) was obtained from a plot between temperature and enzyme activity. At a desired temperature, enzyme activities were also evaluated at different time intervals (10–60 min). The time for half enzyme inactivation ($T_{1/2}$) was also calculated, as described for $T_{1/2}$.

Results

Co-expression of PfOPRT–PfOMPDC complex

The PfOPRT–PfOMPDC was expressed in E. coli as an enzyme complex. Following Ni²⁺-NTA agarose affinity and Hi Trap Q HP anion-exchange chromatography, major PfOPRT and PfOMPDC activities were found in overlapping peaks, i.e., at 50 mM Tris–HCl pH 8.0 with 250 mM NaCl (Fig. 1). The enzyme complex purity was more than 95%, as assessed by SDS–PAGE (Fig. 1, Inset). The recombinant active enzyme complex had specific activities of 8.1 and 29.1 μmol min⁻¹ mg protein⁻¹ for each of the PfOPRT and the PfOMPDC components, respectively, in which the protein ratio of both components in the complex was approximately at 1:1. The molecular mass of the PfOPRT and PfOMPDC components of the complex was estimated to be 35.6 and 41.5 kDa, respectively.

Kinetic properties of PfOPRT–PfOMPDC complex

$K_m$ values for orotate and PRPP of the PfOPRT component in the complex were higher by 2.9- and 2.0-folds, respectively, than the values in its monofunctional form. The $K_m$ values for orotate and PRPP in the complex were higher by 7.3- and 7.1-folds,
respectively, as compared with the monofunctional form. Catalytic efficiency (\(k_{\text{cat}}/K_{m}\)) values for orotate and PRPP of the PIPORT component in the complex were 20.0 and 14.1 times greater than the monofunctional form (Table 1). In case of the PIPOMDC component in the complex, \(K_{m}\) for OMP, \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_{m}\) were not significantly different from its monofunctional form (Table 2).

To simulate the physiological behavior of the complex, the kinetic parameters of each component were determined while another component had been activated by pre-incubating with its substrates (namely, the activated enzyme complex). The enzyme behavior followed Michaelis kinetic. The \(k_{\text{cat}}/K_{m}\) values of the activated complex were ~two-fold better than the non-activated complex, especially for the PIPORT component in the complex (Tables 1 and 2).

**Inhibition and reaction kinetic mechanism of the enzyme complex**

PIPORT catalyses reversible reaction of orotate and PRPP to OMP and PPI, whereas PIPOMDC catalyses irreversible direction of OMP to UMP and CO₂. A classical inhibitor for OMPDC, 6-aza UMP [6], was the most effective competitive inhibitor for the monofunctional PIPOMDC (\(K_{i} = 0.7 ± 0.04 \mu M\)) and the complex (\(K_{i} = 1.0 ± 0.1 \mu M\)). Surprisingly, 6-aza UMP was likewise found to be an effective inhibitor of the PIPORT component in the complex (Table 3), but did not inhibit the monofunctional PIPORT. PPI, interestingly, was a good inhibitor of the PIPORT component in the complex (Table 3), but did not inhibit the monofunctional PIPORT. PPI had greater inhibitory effect (11.2-fold when orotate concentration was varied; and 125-fold when PRPP concentration was varied) to the PfOPRT component in the complex than to the PfOMPDC component in the complex (Tables 1 and 2).

Product inhibition studies for the forward reaction of the PIPORT component in the complex were analyzed by varying both orotate and PRPP concentrations, with the product OMP was a competitive inhibitor. When varying the orotate concentration and fixing PRPP at a saturating concentration in the presence of the product PPI, a non-competitive inhibition type was observed (Fig. 2A). By varying the PRPP concentration and fixing orotate at a saturating concentration in the presence of PPI, a competitive inhibition type was found (Fig. 2B). Kinetic analysis suggests that the enzymatic catalysis of the PIPORT component in the complex followed a random sequential kinetic mechanism.

**Table 1** Kinetic constants of monofunctional PIPORT (mono), PIPORT component in the enzyme complex (complex), and the substrate-activated enzyme complex (activated complex).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Mono</th>
<th>Complex</th>
<th>Activated complex*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orotate</td>
<td>20 ± 0.8</td>
<td>7.0 ± 0.3</td>
<td>4.7 ± 0.6</td>
</tr>
<tr>
<td>PRPP</td>
<td>1.0</td>
<td>7.3</td>
<td>1.6 ± 10⁶</td>
</tr>
<tr>
<td>OR/PRPP</td>
<td>0.5 × 10⁵</td>
<td>1.0 × 10⁶</td>
<td>1.6 × 10⁶</td>
</tr>
</tbody>
</table>

* The complex had been activated with OMP, the substrate of the PIPOMDC component.

**Table 2** Kinetic constants of monofunctional PIPOMDC (mono), PIPOMDC component in the enzyme complex (complex), and the substrate-activated complex (activated complex).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Mono</th>
<th>Complex</th>
<th>Activated complex*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orotate</td>
<td>16.1 ± 1.0</td>
<td>7.9 ± 0.2</td>
<td>5.1 ± 0.6</td>
</tr>
<tr>
<td>PRPP</td>
<td>1.1</td>
<td>7.8</td>
<td>7.8</td>
</tr>
<tr>
<td>OR/PRPP</td>
<td>0.7 × 10⁵</td>
<td>9.5 × 10⁵</td>
<td>1.5 × 10⁶</td>
</tr>
</tbody>
</table>

* The complex had been activated with both orotate and PRPP, the substrates of the PIPORT component.

**Table 3** Inhibition and reaction kinetic mechanism of monofunctional PIPORT (mono) and the PIPORT component in the enzyme complex (complex).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Monofunctional PIPORT (mono)</th>
<th>PIPORT component in the enzyme complex (complex)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitor type</td>
<td>Competitive</td>
<td>Non-competitive</td>
</tr>
<tr>
<td>Mono 6-aza UMP</td>
<td>7.0 ± 0.3</td>
<td>No effect</td>
</tr>
<tr>
<td>Complex 6-aza UMP</td>
<td>50.8 ± 3.9</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Mono PRPP</td>
<td>7.9 ± 0.3</td>
<td>No effect</td>
</tr>
<tr>
<td>Complex PRPP</td>
<td>43.5 ± 3.1</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>Inhibitor: PPI</td>
<td>18.2 ± 0.9</td>
<td>79.7 ± 5.1</td>
</tr>
</tbody>
</table>

* \(K_{m}\) was determined as \(K_{m}\) value of the enzyme in the presence of inhibitor.

**Discussion**

In *P. falciparum*, ORP and OMPDC form an \(\alpha_2\beta_2\)-like enzyme complex as (PIPOR)₂(PIPMDC)₂ [14,16]. The genes of both enzymes are co-expressed in *E. coli*. The recombinant enzyme is co-purified as a complex in the chromatographic steps used. Similar kinetic and physical properties were obtained when compared to the native enzyme complex purified directly from the parasite [16]. The catalytic and kinetic parameters (i.e., \(K_{m}\), \(k_{\text{cat}}\), catalytic efficiency) of the recombinant enzyme complex are more effective than those values of the recombinant monofunctional forms. The kinetic properties of the complex are similar to the other enzyme complex system reported, such as in Trichoderma longibachiatum hemicellulase [19], Leishmania major dihydrofolate reductase-thymidylate synthase [20–22], Salmonella typhimurium pyruvate dehydrogenase [23] and tryptophan synthase [24]. The catalytic efficiency of the substrate-activated PIPORT–PIPMDC complex is better than the non-activated enzyme complex, however allosteric kinetic behavior of the complex was not elucidated. The result demonstrates that when either the PIPORT or the PIOMDC components of the complex is activated, the complex is structurally changed for an efficient binding to their substrates.
phenomenon is an important feature of catalysis in the enzyme complex. That should be further explored.

The enzymatic reaction mechanism of PfOPRT component of the complex follows a random sequential kinetic type, as found previously in the monofunctional form [15]. Our results show that when the enzyme complex is formed, the reaction kinetic mechanism of the enzyme is maintained. This observation is similar to S. typhimurium OPRT [25], although the yeast enzyme displays a sequential mechanism order, where binding for PRPP comes first and orotate comes last [26]. In the presence of 6-aza UMP, $K_m$ values for orotate and PRPP of the PfOPRT component in the complex are 7.3- and 5.5-folds greater, respectively, than those obtained using the monofunctional form. Furthermore, 6-aza UMP has no any inhibitory effect in the monofunctional PfOPRT, but shows good inhibition for the PfOPRT component in the complex, as well as to the monofunctional PfOMPDC. It is thus suggested that 6-aza UMP is a strong competitive inhibitor for both enzymes in the complex. This also implies that the active site of both PfOPRT and PfOMPDC components in the complex may lie near each other, where 6-aza UMP binding to the active site of the PfOMPDC component can interfere to the binding of the substrate orotate and PRPP to the PfOPRT component. However, this evidence needs to be further verified using the crystal structure of the enzyme complex. At present, we have elucidated only the crystal structure of the monofunctional PfOMPDC, and the proposed structure may participate in making the complex with PfOPRT [27].

The product P Pi is a very weak inhibitor of the monofunctional PfOPRT, but is a powerful inhibitor (120-fold difference) of the PfOPRT component in the complex. Comparing the $K_i$ values with other inhibitors of OPRT, $K_i$ for P Pi is the lowest for the enzyme complex. However, the inhibitory effect of P Pi in P. falciparum culture have not yet been performed. Considering the kinetic parameters, e.g., $K_m$ and $K_i$, P Pi and/or its analogs might be candidate for the effective antimalarial drug. Our conclusion is well supported by a recent data on p-nitrophenyl riboside analogue of PRPP shown as an effective inhibitor for the PfOPRT enzyme [28].

Potentially, the PfOPRT–PfOMPDC complex tolerates thermal inactivation better than the monofunctional PfOPRT and PfOMPDC. Formation of the PfOPRT–PfOMPDC as the complex may protect their active sites from thermal inactivation. This characteristic might explain the functional role of the PfOPRT–PfOMPDC complex to maintain the enzymatic integrity during the parasite multiplication in human blood, in lines of the situation that infection results to heat generation as manifested by fever ($\sim$40–41 °C) in human malarial patients. It is also noted that the $K_m$ value of the complex at temperature of 50% inactivation is much higher than that of the $K_m$ at 37 °C, implying enzyme inactivation at high temperature due to the structural change and decrease in substrate binding.

In summary, the functional PfOPRT–PfOMPDC enzyme complex expressed in E. coli exhibits significant enzyme kinetic benefits and thermal stability better than the monofunctional PfOPRT and/or PfOMPDC. P Pi and 6-aza UMP are very good inhibitors for the
enzyme complex. This unique property, thus, raises the possibility of a new drug target for the PPOPT–PROMDC enzyme complex, which can be further exploited for a rational drug design approach for more effective antimalarials [29]. Antimalarial drug development is of utmost importance considering the increased resistance to current first-line antimalarial drug [30].

Acknowledgments

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